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An investigation into the use of fungicides for the control of *Leptosphaeria maculans* and *L. biglobosa* in New Zealand *Brassica napus* ssp. *napobrassica* crops

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A Dissertation

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by

Dayne Barry Paton

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Abstract of a dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Agricultural Science with Honours.

An investigation into the use of fungicides for the control of *Leptosphaeria maculans* and *L. biglobosa* in New Zealand *Brassica napus* ssp. *napobrassica* crops

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Three experiments were conducted to assess the efficacy of six fungicides to inhibit mycelial growth and conidial germination of *Leptosphaeria maculans* and *L. biglobosa* on swede crops in New Zealand. In experiment one, mycelial discs were grown on agar amended with azoxystrobin, epoxiconazole, carbendazim, fluquinconazole, flusilazole and iprodione at a range of concentrations for 12 days. Experiment two involved placing a 50 µl drop of conidial spore suspension on the amended agar and counting the percentage of spores which germinated after 45 hours. Experiment three involved azoxystrobin, fluquinconazole and epoxiconazole applied at field rate to a susceptible and resistant swede variety inoculated with conidial spore suspensions.

Mycelial growth EC_{50} ranged from 0.152 mg a.i. / L to greater than the maximum concentration applied (3.0 mg a.i. / L). Azoxystrobin provided the greatest control, with epoxiconazole and flusilazole also effectively inhibiting growth of the isolates. Iprodione did not inhibit 50% of the growth at the concentrations tested and was not continued for experiment two. Germination inhibition EC_{50} values for the fungicides ranged below and above the minimum and maximum concentrations tested. Azoxystrobin provided the greatest conidial germination inhibition (EC_{50}: <0.003 mg a.i. / L), with epoxiconazole, fluquinconazole and flusilazole ranging from 0.028 to 0.224 mg a.i / L EC_{50} for *L. maculans*. Carbendazim did not reach 50% inhibition at the concentrations tested. Azoxystrobin, epoxiconazole and fluquinconazole inhibited *Leptosphaeria* spp. infection of both the resistant and susceptible swede varieties in the pot experiment.

This work has provided information on the potential to control both *Leptosphaeria maculans* and *L. biglobosa* infection of swede in New Zealand using fungicides, which warrant further testing under field conditions.

Keywords: *Leptosphaeria maculans, L. biglobosa,* mycelium, conidial germination, *in vitro,* EC_{50}, fungicide, *Brassica napus* ssp. *napobrassica*, resistant, susceptible, pot experiment.

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Chapter 1

Introduction

Forage brassicas including kale, turnip, forage rape and swede are important crops for many New Zealand farms (Westwood & Mulcock, 2012). Swedes are the second most commonly grown brassica behind turnips with the majority of them grown in Southland (Andreucci, 2013). It is the favoured option for winter feed in some areas due to its high quality bulb and yields which can reach up to 20 t/ha, with 15 t/ha crops common on good soils. They perform best in moist, cool environments and have a low drought tolerance so are typically grown in Southland and Otago but are also grown in the central North Island. They are commonly grazed from early to late winter with the bulbs keeping well but as the season progresses the quality of the leaf declines due to adverse weather and disease. The plant is typically made up of 20-30% leaf and 70-80% bulb with a white or yellow flesh (Stewart *et al*. 2014). Westwood and Mulcock (2012) found the average megajoules of metabolisable energy per kilogram of dry matter (MJME/kg DM) of six varieties of swede was 13.8 MJME/kg DM with an average digestibility of 93.5%. This was much higher than the other brassicas tested such as kale which averaged 11.2 MJME/kgDM and 77% digestibility, or bulb turnip which averaged 11.7 MJME/kgDM and 89% digestibility. Due to their susceptibility to disease it is recommended to not sow a second crop of swedes in the same paddock. Once established, the main threats to swedes are dry rot (*Leptosphaeria spp.*), club root (*Plasmodiophora brassicae*) and aphids (*Brevicoryne brassicae*) (Stewart *et al*. 2014). This research project focuses on dry rot of swedes in New Zealand and the potential of fungicides to reduce disease incidence.

Dry rot (Black leg) is caused by *Leptosphaeria maculans* and *L. biglobosa* on a variety of brassica species including oilseed rape, turnips, cabbage and swedes, as well as other weed species. Dry rot is distributed worldwide and is most serious in temperate areas where the disease thrives (Rimmer *et al*., 2007). The first report of dry rot in New Zealand was by G.H. Cunningham in 1927 when over 400 isolates of *L. maculans* were identified as the anamorph *Phoma lingam* recovered form diseased swede and turnip crops. Before 2000 it was believed only *L. maculans* was the cause of black leg and dry rot with isolates defined as being one of two pathotypes, A (highly virulent) and B (weakly virulent). Subsequently based on differences in the sequence of taxonomic genes (ITS, β-tubulin and actin) these two pathovars were distinguished as being separate species and *L. biglobosa* was described (Voigt *et al*., 2005). *Leptosphaeria maculans* has been identified as the major cause of dry rot in New Zealand with the prevalence of *L. biglobosa* not largely studied. In a survey conducted by Lob (2014) the colony morphology of fungi isolated from samples of diseased oilseed rape, swede, turnip, kale, rape, broccoli and cauliflower crops from several areas between Southland and Hawkes

Bay with characteristic *L. maculans*/L. *biglobosa* leaf lesions identified 127 isolates of *L. maculans* and only 4 of *L. biglobosa*. While this is only one study, during one year, it shows that *L. maculans* is the dominant species associated with these symptoms in New Zealand. Yet with different weather events each year and changing climates it is possible *L. biglobosa* may show as an important species in future studies. Despite *L. biglobosa* being known for being weakly aggressive (Rimmer *et al*. 2007), Karolewski *et al*. (2002) has reported that in western Poland only *L. maculans* was found to infect oilseed rape, while in eastern Poland only *L. biglobosa* was found. This may be important as changes in climate could favour *L. biglobosa* and therefore control strategies which are effective against both species need to be implemented.

Dry rot is the most important disease of swedes due to its ability to almost completely destroy the crop and make the bulbs unfit for grazing (Gowers & Armstrong, 1997). Due to the relatively small scale of forage swede there has been little research regarding the control of dry rot despite its importance to many farmers in New Zealand as a high quality winter feed. Oil seed rape (Canola) is a very economically important host of *Leptosphaeria maculans and biglobosa* which has had much more research focused towards it. The pathogens are reported to infect and cause disease in swede in a similar way to that reported for oilseed rape. Due to the limited research on swede, earlier research will rely on examples of the disease cycle and control strategies reported for *L. maculans* and *L. biglobosa* on oilseed rape. This shows that there is potential for more research on dry rot in swedes to be carried out to allow farmers to make better informed decisions about their integrated pest management solutions. The following literature review will cover the options currently available to New Zealand farmers to control dry rot in forage swedes and options which may be adopted in the future.

Chapter 2

Literature review

2.1 Introduction

The objective of this literature review was to gain a better understanding of dry rot in swedes and the options available to farmers for its control with particular focus on how fungicides can be used as a part of an intergrated pest management plan.

2.2 Disease cycle

The worldwide distribution of *L. maculans* and *L. biglobosa* is believed to be due to their transmission within seeds (Lob, 2014). The fungus may survive for years as dormant mycelium on infected seeds and is thought to have spread around the world in this way (Rimmer *et al*., 2007; Harvey, 2010). In 1940, New Zealand began certifying seed through the then Department of Agriculture as 'certified' and 'government approved' seed. Since then most seed has been tested and is almost always disease free, any seed lots which are infected rarely exceed 0.1% infection (Allen & Smith, 1961). Infested stubble is the major source of inoculum for spread between seasons and between areas. This is because the disease can live in plant material in the soil as long as the crop residues remain which can be up to 4 years if there is a series of dry years (Harvey, 2010; Fernando *et al*, 2007). This period of dormancy can vary due to the conditions causing the breakdown of the stubble. For example canola stubble in Australia takes longer to break down than in Europe because of the drier climate (West *et al*., 2001). Once the conditions are favourable, the dormant fungi will begin to produce pseudothecia, these contain ascospores which can become airborne and travel distances of 1-2 kilometres and a maximum of 10 kilometres (Fernando *et al*. 2007). The ascospores are released after humid or wet weather and can remain viable for up to six weeks before infecting the new host. Once on the host the ascospore will infect the host through wounds or stomata without the aid of penetrating structures such as appressorium (Fernando *et al*. 2007). As a lesion establishes itself on the leaf it will begin to produce pycnidia containing conidia which are rain splashed onto other areas of the host plant such as leaves or cracks on the shoulders of the bulbs and neighbouring plants (Figure 2.1). Transfer to the bulbs can also occur when infected leaves fall onto the bulb resulting in direct contact and transfer of the infection (Harvey, 2010). Systemic progression of the fungus down the petioles and into the stem is reported in oilseed rape and kale and has also been shown in swedes (Lob, 2014). The infections on the leaves produce large pale lesions with many pycnidia that appear as small dark spots while infection of the bulb causes dry, brown-black lesions also with abundant conidia (Lob, 2014). After producing large bulb cankers, the fungus then survives in the

infected crop residues until the right conditions present themselves to produce pseudothecia and ascospores which infect the following crop. (Harvey, 2010)

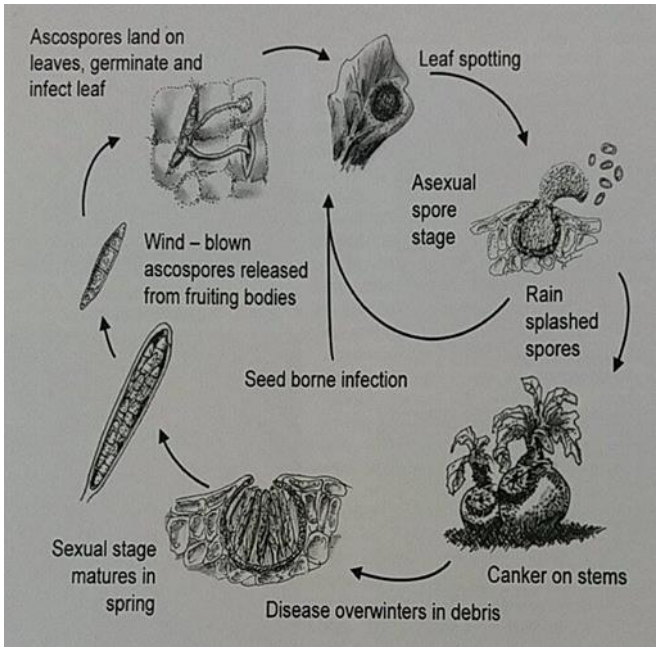

Figure 2.1 Disease cycle of *Leptosphaeria maculans*, the causal agent of dry rot on swede (From Harvey, 2010).

2.3 Control Methods

2.3.1 Cultural

Crop rotation is one of the most effective cultural control strategies for most diseases as it starves the disease of a host. It works by allowing the infected crop residues time to break down in the soil and the disease structures to lose viability while other crops which aren't a host to the disease are grown (Fernando *et al*. 2007). If dry rot occurs in a crop, it is recommended not to sow the paddock back in swedes for four years, as the fungus can survive in crop residues for at least three years (Harvey, 2010). Guo *et al*. (2005) studied the effects of crop rotation and tillage methods on the disease incidence of blackleg on canola and found that crop rotation significantly reduced the disease incidence on mature canola plants (Table 2.1).

Table 2.1 Black leg incidence (%) on mature canola under different crop rotation and tillage regimes in 2001 and 2002, C (Canola), W (Wheat) and F (Flax). (From Guo *et al*., 2005).

	Tillage	
Rotation	**Conventional**	**Zero till**
2001		
CCC	57 aA	81aB
CWC	49 bA	63 bB
2002		
CCCC	49.3 aA	79.3 aB
CWCC	21.5 bA	61 bB
CCWC	16.3 bA	56.7 cB
CWFC	21 bA	18.7 dA

Note. Different lowercase letters within a column for each year indicate significant difference between different rotation systems; different capital letters within a row indicate significant difference between different tillage systems according to the Fisher's protected least significant difference (LSD) test ($P \geq 0.05$)

It is also recommended to plough fields after they have been infected, this speeds up the breakdown of the crop residues which the fungus is relying on to survive. It is most effective if the material is chopped and buried (Harvey, 2010). Turkington *et al.* (2000) found that burying the stubble of oilseed rape into the soil resulted in 40% breakdown of residue compared to 27% breakdown of surface stubble after a one year and three months. Similar results were observed by Lob (2014) where burial of oilseed rape stubble resulted in a 51.5% reduction in stubble weight compared with a 31.5% reduction for the stubble left on the soil surface after 6 months. Further burial reduced pseudothecia development on the stubble after 8 weeks. The results from Guo *et al*. (2005; Table 2.1) also support these findings with treatments under conventional tillage having significantly less disease than those with zero tillage. Although, these studies involved oilseed rape which is likely to breakdown differently due to structural differences in the tissue compared to swede. Additionally, the stubble material was kept in nylon mesh bags for experimental control purposes which may have effected its breakdown. Despite this it is highly likely that further studies involving swedes will produce similar results.

More research studying the effects of crop rotation and ploughing on dry rot in swedes may be useful; however, research from other crops and crop diseases is clear that crop rotation and ploughing reduces disease carry over. With the adoption of minimum tillage becoming very common, ploughing is not a very useful option for the control of dry rot in swedes as most farmers now only have the equipment for minimum tillage. Crop rotation is a method which can easily be implemented as forage swedes are a winter feed and only take up a small proportion of the farm which could form part of a re-grassing program. If a farmer needs to put a forage crop in the same paddock for a second year kale is recommended as it is less susceptible to dry rot than swedes; however, this is only recommended if the amount of dry rot in the previous year's swede crop was minimal (Harvey, 2010).

Although there is little research regarding it, *Leptosphaeria* spp. can also infect brassica weeds such as hedge mustard, wild turnip and shepherds purse (Lob, 2014; Rimmer *et al*. 2007). Due to this, care must be taken to control any weeds which may be able to harbour the disease and pass it onto the next crop that is planted. Buying certified seed is also important to ensure that there are no brassica weed seeds in the product a farmer purchases as this could cause an outbreak of a brassica weed on their farm which can act as a host to spread *L. maculans* between swede crops.

Weather based predictive models for ascospore release have been shown to be useful for predictions of pseudothecia maturation based on temperature and moisture (rainfall) data. Salam *et al*. (2007) tested the 'Improved Blackleg Sporacle' model and found it predicted ascospore release within seven days of the actual event for between 42 and 78% of the data points in Australia, Canada France, Poland and the UK. They also tested the SporacleEzy model which predicted dates within seven days of the actual for 47 to 89% of the data points in the same countries. While these tests were carried out on oilseed rape the results are likely to correlate for ascospore release in swede. This would give farmers another tool to improve their strategies for controlling dry rot. This could include adjusting sowing date and improving the timing of fungicide spraying based on the likelihood of ascospore release. The SporacleEzy model is already being utilised in Western Australia to guide farmers on sowing dates and chemical application timing for oilseed rape crops (Salam *et al*. 2007).

2.3.2 Chemical

Fungicides have the potential to contribute to the control of dry rot in swedes but there are currently no products registered to do so in New Zealand (Harvey, 2010; Novachem Manual, 2017). A limited number of trials have been carried out in New Zealand by industry companies, although the actual results of these are not available a summary from a field days handout has been obtained. Munro (2007) used three fungicides, Proline (prothioconazole), Pristine (boscalid + pyraclostobin) and Galmano (fluquinconazole) to attempt to control dry rot in swede crops. In the first year of the trial the fungicides were able to reduce the level of infection, although the infection levels were so low there was no increase in yield from the control plots therefore the trial was continued for a second year. Figure 2.2 shows that the percentage of bulb infection was reduced in the second year by Proline (≈55%) and Pristine (≈35%), yet the Galmano seed treatment only reduced bulb infection by ≈12%.

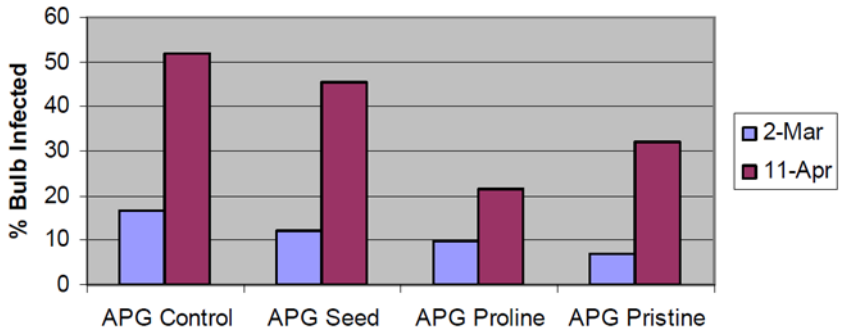

Figure 2.2 Percentage of bulbs infected with dry rot (*Leptosphaeria maculans*) after two applications of Proline and Pristine fungicides or a Galmano seed treatment in a second year crop of Aparima Gold (From Munro, 2007).

The amount of disease present was a good indicator of yield which can be seen in Figure 2.3. The plots treated with Proline and Pristine yielded ≈71% and ≈88% more than the control while the Galmano treated plots only yielded ≈5% more than the control.

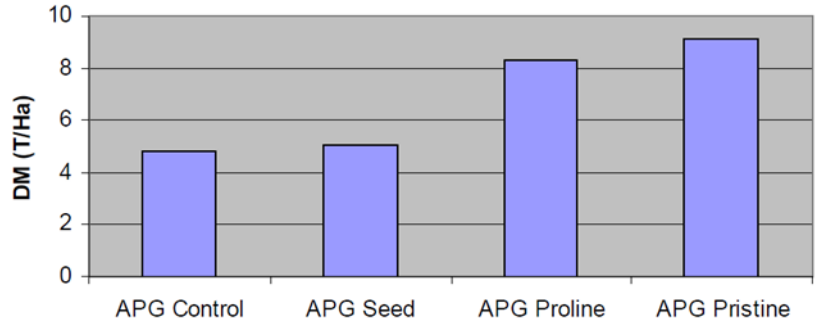

Figure 2.3 Dry matter yield of a second year crop of Aparima Gold after two applications of Proline and Pristine fungicides or a Galmano seed treatment (From Munro, 2007).

This shows that when there is a high level of disease present, such as a second year swede crop, it may be beneficial to apply fungicides in order to control the disease. However, the yields of the treated plots were still relatively low and the cost of fungicides is very high, especially as these trials involved two applications of fungicide at high rates. The seed treatment offered very little control; however, it is a much cheaper and easier option which may give it a role in providing a low level of control for dry rot resistant cultivars (Munro, 2007). This is especially important as forage swedes are not a high value crop so farmers may not have enough in their profit margins to afford to apply expensive fungicides.

There have also been studies looking at the control of blackleg in oilseed rape around the world. In the UK, Huang *et al*. (2011) found that the fungicide Punch C which contains flusilazole and carbendazim was able to reduce the size of leaf lesions on oilseed rape inoculated with *L. maculans* but not *L. biglobosa*. As seen in figure 4, the lesion size of *L. maculans* is reduced to almost nothing by the fungicide treatment 6 days after inoculation, while the treatments inoculated with *L. biglobosa* were not largely effected by any fungicide treatment. This is thought to have been caused by differences in the timing of ascospore release and some resistance to the fungicides but is not considered a major concern as *L. biglobosa* is a much less aggressive species than *L. maculans* (Rimmer *et al*. 2007).

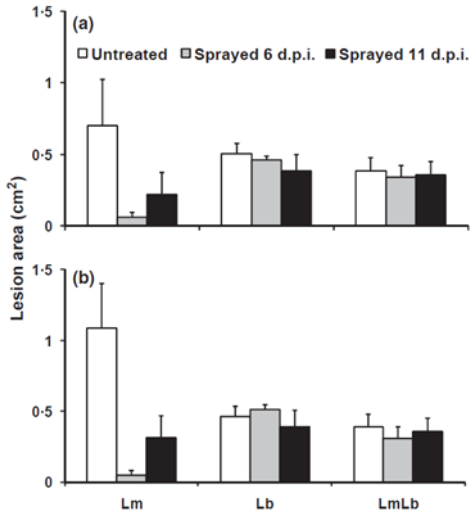

Figure 2.4 : Effects of the fungicide Punch C (flusilazole plus carbendazim) on development of lesions caused by *Leptosphaeria maculans* and/or *L. biglobosa* ascospores on leaves of oilseed rape (Brassica napus) cvs Courage (a) or Canberra (b). There were three fungicide treatments: untreated, or sprayed at 6 or 11 days post-inoculation (d.p.i.). (From Huang et al. 2011).

Huang *et al*. (2011) and Munro (2007) have shown that *L. maculans* is sensitive to fungicides and that with continued research they may become a key tool in the control of dry rot in New Zealand. However, it isn't simply a case of applying a fungicide spray, to get the most out of the fungicides they need to be applied at critical times. This is shown in Figure 2.4 with the spray application applied six days after inoculation providing greater control compared to eleven days. Harvey (2010) suggests that the most effective control strategy is to identify the timing of the leaf infection and spray fungicide to prevent leaf infection and ultimately stop the spread of the disease to the bulb. This requires physically visiting the paddock several times and looking for signs of infection; however, once infection is identified the pathogen has already begun attacking the plant and may not be able to be controlled. With the development of weather based predictive models it is possible to predict ascospore release with some degree of accuracy (Salam *et al*. 2007). This could allow farmers to

apply fungicides to coincide with ascospore release and attempt to stop or reduce the initial infection of the young plants rather than having to monitor for leaf infection (Huang *et al*. 2011). This would also reduce the labour required for crop monitoring; however, the models accuracy would have to improve as the average accuracy for ascospore release within 7 days is only 60% (Salam *et al*. 2007). Marcroft *et al*. (2005) showed that oilseed rape in south-eastern Australia did not develop stem cankers if they were inoculated with *Leptosphaeria maculans* after the three to five leaf growth stage. This could be the case for swedes and if so, would mean farmers would only need to focus on dry rot prevention for the first four to six weeks of the crop reducing the labour requirements of monitoring it and the need for fungicides after this point.

It has been suggested that the application of chemicals such as glyphosate and nitrogen onto crop residues may reduce inoculum production by speeding up residue breakdown and reducing the *L. maculans* population. Turkington *et al*. (2000) assessed the application of glyphosate and nutrients on crop debris breakdown and inoculum production by *L. maculans*. They had four treatments; (1) liquid N at 45 kgN/ha + Glyphosphate at 45 kg C/ha, (2) Urea at 45 kgN/ha, (3) Roundup at 7L of product/ha and (4) Stubble digest all powder (Commercial nutrient medium to enhance microbial activity) at 0.6 kg product/ha. None of the treatments enhanced the breakdown of crop residues or reduced *L. maculans* inoculum production. Similar results were found by Lob (2014) with urea application not increasing the rate of decomposition of stubble; however, in combination with burial, it was shown to reduce pseudothecia development with no pseudothecia recovered from the stubble after 17 weeks. Replications of these experiments using swedes is required; however, it is expected that similar results will be obtained with *L. maculans* infected swede crop debris.

Chemically induced host resistance to pathogens has been shown to be effective in controlling many crop diseases. Liu *et al*. (2006) studied the effects of two of these chemicals; acibenzolar-*S*-methyl (ASM) and menadione sodium bisulphate (MSB) on the resistance of oilseed rape to *L. maculans*. While both chemicals reduced leaf spotting and lesion development later in the season; ASM was significantly more effective than MSB. If these results can be replicated for dry rot in New Zealand swedes, ASM may be a good option if it can be manufactured and applied at an economic rate. Although, it would be difficult to decide if it is necessary each year as it needs to be applied before there is any threat of *L. maculans* ascospore production to allow the plant to respond.

2.3.3 Biological

There is potential for biological control using saprophytic microorganisms which are normally associated with the breakdown of crop residues. Application of these microbes or manipulation of the conditions which favour their activity could be used to increase the rate of residue breakdown and hence reduce the survival of *L. maculans*/*L. biglobosa* inoculum (Turkington *et al*. 2000). This is

already done with the ploughing of fields to incorporate the crop debris and increase soil aeration and therefore microbial activity. There may be an opportunity for the development of a microbial inoculum which can be applied to soil and stubble in conjunction with ploughing; however, more research is needed in this area.

There have been several studies on the inoculation of oilseed rape with *L. biglobosa* ascospores to induce local and systemic resistance to the more virulent *L. maculans* species. This was shown by Liu *et al*. (2006) who inoculated oilseed rape leaves with *L. biglobosa* ascospores in the UK. The treated plants had less leaf spotting in Autumn and a lower incidence and severity of stem cankers 8 months after the treatment. It is thought that this occurs as the pre-treatment allows the plant to build up structural and chemical defence mechanisms such as cell wall mechanisms, salicylic acid and defensive proteins before exposure to *L. maculans*. Treatment with *L. biglobosa* was more effective than the MSB treatment but less effective than the ASM (Liu *et al*. 2006). Research on this method of treatment for control of dry rot in swedes in New Zealand would be beneficial to add another option for farmers if it can be shown to have a positive effect without causing significant disease from *L. biglobosa.*

2.4 Evaluation of control methods

Crop rotation is first and foremost the best tool a farmer can adopt for the control of dry rot which doesn't have to cost the farmer anything providing the logistics of paddock selection allow for it. If a farmer can easily plough a field this is also highly recommended as it speeds up the breakdown of crop residues through burying them. By adopting these two methods as well as controlling brassica weeds and using certified seed, farmers will reduce the risk of a major break out of dry rot on their farm without making any major changes or spending lots of money. Sometimes infection will be inevitable and other methods will need to be adopted. While most of the other options have little research focused on dry rot in swedes they have been proven on the same pathogen (*L. maculans*) in oilseed rape. These include fungicides, chemical induced resistance, *L. biglobosa* induced resistance, saprophytic micro-organisms to breakdown crop residues and weather based predictive models.

Traditionally, pests and diseases have been controlled through the use of pesticides. With increasing pressure on farmers to reduce the inputs into their farms and maintain the clean and green image, pesticide use is declining. In the case of swedes, fungicides have not been registered for use in New Zealand as the cost is too high for the relatively low value forage crop. As we learn more about the disease and the effectiveness of potential fungicides on swedes, their use may become more common as a part of an integrated pest management program. Currently, seed treatments have the most value as they are cheap and could offer background protection for new dry rot resistant cultivars. The role of fungicides will not be for blanket sprays every season, they will be utilised at

critical times during bad seasons where the conditions are supporting a dry rot outbreak. Crop monitoring and weather based predictive models will assist farmers in deciding when fungicides might be needed.

Other chemical options are acibenzolar-S-methyl (ASM) and menadione sodium bisulphate (MSB), these have been shown to produce resistance to black leg (*L. maculans*) in oilseed rape. These studies could be reproduced for dry rot in swedes to test their effectiveness although, they are unlikely to be useful as they will be required to be sprayed every year before knowing the disease pressure for that season. The biological control in the form of *L. biglobosa* has been shown to induce blackleg resistance in oilseed rape which would be a great alternative for sustainable farming. If this can be proved to work for dry rot in swedes it will be a great opportunity to add another option for farmers in the fight against dry rot before they have to resort to chemical use, although it will have to be shown to not induce any economically significant disease itself.

2.5 Conclusion

Currently there are not many options for farmers growing swedes to combat dry rot apart from cultural methods. While this is an issue, if implemented correctly the cultural methods should minimise any effects of dry rot infection in their crops for the majority of seasons. In the future with continued work testing fungicides, as well as chemical and biological resistance inducers coupled with weather based predictive models, farmers will have more options to reduce the impact of dry rot infection in their crops.

Chapter 3

Materials and Methods

3.1 Objectives

The objective of this honours project was to determine the potential for control of *L. maculans and L. biglobosa* infection of swede crops in New Zealand using fungicides.

3.1.1 Objective 1: *In vitro* sensitivity *of Leptosphaeria maculans* and *L. biglobosa* to selected fungicides.

To determine the sensitivity of mycelial growth and conidial germination from different isolates of *Leptosphaeria maculans* and *L. biglobosa* to a range of fungicides by determining the EC_{50}.

3.1.2 Objective 2: Assessing the effect of selected fungicides to prevent infection of swede by *L. maculans* and *L. biglobosa*

To determine the effectiveness of three fungicides with the lowest EC_{50} values for both mycelial growth and conidial germination of the *Leptosphaeria maculans* and *L. biglobosa* isolates applied at field rate to prevent infection of a dry rot resistant and dry rot susceptible swede cultivar inoculated with conidial spore suspensions.

3.2 Effect of fungicides on *in vitro* mycelial growth of *Leptosphaeria spp.*

Two *L. maculans* (LM183 and LM145) and one *L. biglobosa* (LB237) isolate, recovered from symptomatic brassica tissue were obtained from the Plant Pathology culture collection, Lincoln University. The *L. maculans* isolates were selected to represent the population recovered from dry rot in swedes. There were no *L. biglobosa* isolates recovered from swede by Lob (2014) therefore an isolate recovered from kale was selected. All isolates were stored as mycelial discs in 20% glycerol - 80^{o}C and routinely sub cultured onto potato dextrose agar (PDA; DifcoTM, New Jersey, USA) plates for multiplication. After 14 days at 20°C in 12 hours light and 12 hours dark, 5 mm diameter plugs were taken from the colony margin and transferred to 10 new PDA plates for multiplication and grown for a further 14 days.

Table 3.1 Details of fungicides used in the experiments

Active ingredient	Trade name	Chemical class	Conc. range (mg a.i / L)	Mode of action
Azoxystrobin	Amistar	Strobilurin	0.003 - 3.0	Systemic, translaminar, protectant & anti sporilant
Epoxiconazole	Opus	Triazole	0.003 - 3.0	Contact, systemic, protectant & eradicant
Carbendazim	Protek	Benzimidazole	0.003 - 3.0	Protectant, eradicant and locally systemic
Fluquinconazole	Jockey	Triazole	0.003 - 3.0	Systemic, contact, residual, disinfectant & preventative
Flusilazole	Megastar	Triazole	0.003 - 3.0	Inhibitory & true systemic
Iprodione	Rovral	Dicarboximide	0.003 - 3.0	Contact & limited eradicant activity

Note. (Novachem Manual, 2017)

Six fungicides belonging to different chemical groups (Table 3.1) were tested *in vitro* to determine their effect on inhibiting mycelial growth and conidial germination of *L. maculans* and *L. biglobosa* based on reported efficacy against *L. maculans*/*L. biglobosa* in the literature, or those registered for use on brassica (Eckert *et al*., 2010; Novachem Manual, 2017; Ratajkiewic *et al*., 2009; Huang et al., 2011). Each fungicide was tested at four concentrations (0.003, 0.03, 0.3 and 3 mg a.i. / L) which were chosen based on the reported activity ranges. Fungicide amended agar plates were produced by adding 250 mL of double strength fungicide (0.006, 0.06, 0.6 or 6 mg a.i. / L) to 250 mL of double strength agar (78 g/L). The agar was cooled down to 50°C so that the fungicide would not be degraded and the fungicide solutions were warmed slightly so the agar would not set before it was poured into the 90mm Petri dishes. Each of the *Leptosphaeria* spp. isolates and fungicide concentration treatments had five replicate plates. Unamended PDA control plates were included for each fungicide and *Leptosphaeria* spp. isolate combination. Plugs (5 mm diameter) were taken from the margin of a *L. maculans* or *L. biglobosa* colony growing on a PDA plate and arranged in the centre of the appropriate fungicide amended plates. The plates were arranged in a complete randomised design in an incubator at 20°C with 12 hours light and 12 hours dark. The colony diameter was measured using a digital calliper (Mitutoyo, Kanagawa, Japan) after 8, 12 and 16 days growth and recorded in excel.

The day 12 data was chosen for further analysis as it was the period with the greatest growth without growing to the edge of the Petri dishes. The average of the two perpendicular measurements of each plate were taken and calculated as a percentage of the corresponding control plate. These values were then transformed to Log_{10} values and used to calculate the EC_{50} (the effective fungicide concentration which reduced mycelial growth below 50%) using Probit analysis in

the GenStat 18 software. The mean EC_{50} values were then analysed using the ANOVA software in Genstat18 along with a fishers protected LSD to compare differences within the data (P=0.05).

The isolates or fungicides which had an EC_{50} greater than the highest concentration tested in the experiment (3.0 mg a.i. / L) were given an 'MC' rating, meaning maximum concentration. This is because concentrations greater than 3.0 mg a.i. / L were not tested so a reliable $EC5_{50}$ value could not be calculated. The same principle was used for minimum concentration, with EC_{50} values less than the minimum concentration tested (0.003 mg a.i / L) given a 'mc' result. The EC_{50} results above the minimum and maximum were still used for the purpose of the statistical analyses.

3.3 Effect of fungicides on *in vitro* conidial germination of *Leptosphaeria* spp. conidia

Iprodione was not continued for the germination experiment due to its poor performance in the mycelial growth inhibition experiment. For the remaining five fungicides the same concentrations (0.003 – 3.0 mg a.i. / L) were used with the plates produced as described in Section 3.2. The *L. maculans* isolates LM183 and LM145 and *L. biglobosa* isolate LB237 were also used again. Four replicates of each treatment were used in this experiment.

The methods described by Lob (2014) were followed for the production of spore suspensions for each *Leptosphaeria spp.* isolate fresh on the day of each experiment. The spore suspensions were produced from *Leptosphaeria spp*. isolates grown on PDA and incubated under a light bank (continuous cool white fluorescent light (100-150 µE.s-1.m-2)) at room temperature (20-31°C) for 16 days. These incubation conditions were reported by Lob (2014) to provide optimum conditions for pycnidial growth and hence conidial spore production. The incubated plates were then flooded with 10mL of sterilised distilled water amended with one drop/100mL of Tween 80. The surface of the plate was rubbed gently using a hockey stick rod and left to sit for 20 minutes to allow the pycnidia to absorb water. The suspension was then strained through Miracloth to remove mycelial fragments and the conidia count was measured using a haemocytometer. The suspension was then diluted to a spore count of 10^6 conidia/ml for plating.

Two 50 µL drops of the spore suspension were placed on each fungicide amended agar plate which were then incubated at 20°C for 45 hours to allow germination. Unamended PDA plates were used as controls and four replicate plates were set up for each isolate/fungicide treatment combination. After 45 hours incubation a 5mm x 5mm square of agar was cut randomly from one of the two drops of spore suspension and placed on a glass slide. A drop of lactophenol cotton blue was applied and a cover slip was placed over the top. All of the treatments and replicates were prepared before counting the spores as the cotton blue inhibited further germination. Random groups of spores were

then counted to a total of 100 using handheld counters, one to count the total number of spores and one to count the germinated spores. A spore was considered germinated if its germ tube was longer than half the width of the spore (Figure 3.1).

The *L. biglobosa* isolate conidial germination plates were incubated for 20 hours which was four hours longer than the optimum time according to lob (2014). After experimenting with the *L. maculans* isolates it was determined the optimum incubation was 45 hours but at this time *L. biglobosa* could not be repeated. The *L. maculans* and *L. biglobosa* isolates were then analysed separately.

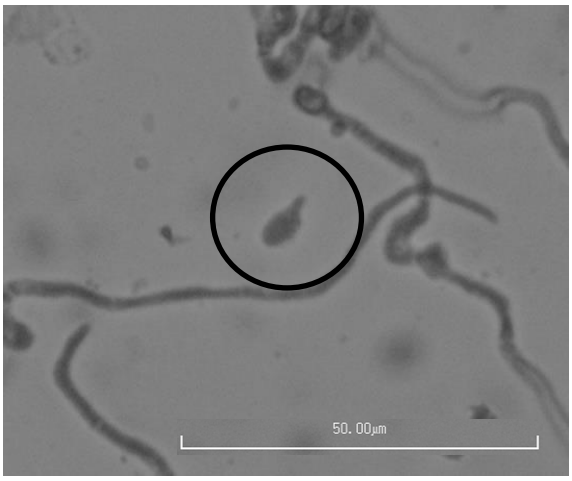

Figure 3.1 Conidial spore from *Leptosphaeria maculans* isolate LM183 showing germ tube half the width of the spore.

The results were recorded in excel and converted to Log_{10} values to calculate EC_{50} values in Genstat18 using the probit analysis software. The mean EC_{50} values were then analysed using the ANOVA software in Genstat18 along with a fishers protected LSD to compare differences within the data (P=0.05). EC_{50} results greater than 3.0 mg a.i / L or less than 0.003 mg a.i / L were given a maximum or minimum concentration result as described in section 3.2.

3.4 Swede seedling inoculation pot experiment

A pot experiment was conducted to determine the effectiveness of the top three fungicides from the mycelial and germination tests to inhibit infection by *L. maculans* and *L. biglobosa*. These were azoxystrobin, epoxiconazole and carbendazim which were made up to field rates (Table 3.2). As there are no fungicides registered for use on swedes in New Zealand, field rates were based on recommendations for wheat found in relevant sources.

Table 3.2 Field application rates of the fungicides used in pot experiment and the source which the field rate was obtained from.

Fungicide	Field rate (ml/L)	Source
Azoxystrobin	6.67	DPIRD. (n.d.).
Epoxiconazole	15	Novachem Manual. (2017).
Fluquinconazole	3	Metcalfe *et al*. (2000)

The pot experiment was set up using the method described by Lob (2014). Two swede cultivars were obtained from Agriseeds one of which is reported to be dry rot resistant (WTNTCIOUH) and one dry rot susceptible (DMNDOMIOTT) (Jenny Barrett, Agriseeds, Pers. Comm.). The swede seedlings were grown in 10 cm diameter plastic pots filled with 3-4 month potting mix. This contained 400 L of horticultural bark (grade 2), 100 L pumice grade 1-4 mm, Osmocote exact N:P:K (16:3.5:10) 1500g, horticultural Lime 500g and Hydraflo 500g. Four swede seeds were planted in each pot, 1 cm below the surface and placed in the greenhouse. Once emerged, the seedlings were thinned to one seedling per pot, randomly assigned a treatment group and placed into a complete randomized block design. The treatments included three fungicides and two pathogen treatments as isolate suspensions (*L. maculans* and *L. biglobosa*) as well as a positive and negative control. The isolate suspensions were made as described in Section 3.3 with an equal mixture of LM183 and LM145 used for *L. maculans,* both were diluted to 10^5 conidia/ml. Once the seedlings were 10 days old the fungicide treatments were applied to the cotyledons till runoff occurred. Once the fungicides had dried a cotyledon was randomly chosen from each seedling and wounded using a sewing needle with a piece of styrofoam behind for support. A 10 µl drop of the appropriate isolate suspension or sterile water was then placed on the wound. Both the positive and negative controls consisted of plants sprayed with sterile water instead of fungicide. The positive controls were inoculated with *L. maculans* or *L. biglobosa* spore suspensions and the negative controls were inoculated with sterile water. Eight replicates of each treatment were set up. After the inoculum had dried, a plastic bag was sprayed with sterile water on the inside and placed over the pot to maintain 100% RH for 72 hours to provide optimum conditions for conidial germination and subsequent infection. The plants were arranged in a completely randomised design and watered every second day with a beaker so not to wash the leaves.

After 15 days, disease incidence was measured using the scale of 0-9 from Lob (2014) where 0: no visible symptom; 1: necrotic hypersensitive; 2: grey-green tissue collapse (1 mm diameter) with a distinct margin; 3: collapsed spots (2 mm diameter) with a distinct margin; 4: collapsed spots (2-3 mm diameter) with a diffuse margin; 5: collapsed spots (3-4 mm diameter) with a diffuse margin; 6: collapsed spots (5-6 mm diameter) with a diffuse margin; 7: collapsed spots (>6 mm diameter) with a diffuse margin and a few pycnidia; 8: collapsed cotyledon tissue with a few pycnidia; 9: collapsed

cotyledon tissue with masses of pycnidia. Lesion diameter was measured using a digital calliper in two perpendicular directions. The data was analysed using ANOVA in Genstat18 with a fishers protected LSD to compare differences between the means.

The plants which weren't used for Koch's postulates (replicates 1-5) were left in the green house to grow for a further 70 days to allow any further disease development to occur. On day 71, the plants which showed signs of disease development were recorded and samples were taken for Koch's postulates to confirm *Leptosphaeria* spp. was the cause.

3.4.1 Koch's postulates

To confirm that the lesions which developed at the inoculation sites were caused by *Leptosphaeria* spp. Koch's postulates were carried out using the methods described by Lob (2014). Small samples of inoculated cotyledon (5mm x 10 mm) were taken from replicates 6, 7 and 8 of the positive control treatments and replicate 8 of the other treatments. These were surface sterilised by soaking in sodium hypochlorite solution (0.2% available chlorine) for 1 minute. The plant tissue was then rinsed in sterile water for 1 minute and dried on sterilised filter paper. Once dry the samples were placed in individual Petri dishes containing PDA amended with streptomycin (5 mg/mL) and penicillin (5 mg/mL). The petri dishes were incubated at 15°C in the dark for 15 days and then analysed for their colony morphology to confirm whether *L. maculans* and *L. biglobosa* was the cause of the leaf lesion. Koch's postulates were also used for any plants exhibiting signs of disease at the 70 day assessment.

Chapter 4

Results

4.1 Effect of fungicides on *in vitro* mycelial growth of *Leptosphaeria spp.*

Mycelial growth of all three *L. maculans and L. biglobosa* isolates were inhibited by most of the fungicides tested. There was a significant effect of both fungicide and isolate, and an interaction between them on the EC_{50} value (P<0.001 for all; Table A.1). Across all isolates, the most effective fungicide was epoxiconazole (Figure 4.1; Table 4.1) with an EC_{50} of 0.152 mg a.i / L but it was not significantly greater than fluquinconazole which had an EC_{50} of 0.248 mg a.i / L. Fluquinconazole also shared significance with flusilazole which had an EC_{50} of 0.336 mg a.i. / L. Iprodione was significantly less effective at inhibiting mycelial growth compared with the other fungicides with an EC_{50} value of >3.0 mg a.i. / L. Azoxystrobin and carbendazim also performed poorly with EC_{50} values >1.0 mg a.i. / L. Across all fungicide treatments, the most susceptible isolate was *L. maculans* LM145 which had a significantly lower EC_{50} than both *L. maculans* LM183 and *L. biglobosa* LB237, with the mycelial growth of L*. biglobosa* LB237 being significantly more sensitive than that of *L. maculans* LM183 (EC_{50} of 1.702 and 2.599 mg a.i. / L, respectively).

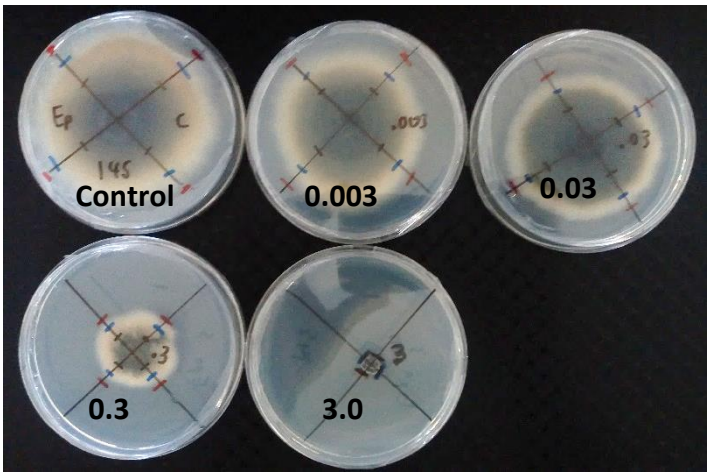

Figure 4.1 Effect of epoxiconazole at four concentrations (inset, mg a.i. / L) on *in vitro* mycelial growth of *Leptosphaeria maculans* isolate LM145 after 12 days growth on amended agar compared with the unamended control.

Table 4.1 The mean EC_{50} values (mg a.i. / L) of different fungicides for the *in vitro* effect on mycelial growth of two *Leptosphaeria maculans* isolates (LM145 and LM183) and one *L. biglobosa* isolate (LB237) relative to the no fungicide control after 12 days incubation at 20°C with 12 hours light.

	***Leptosphaeria spp.* isolate**						**Fungicide mean EC_{50}**	
	LM183		**LM145**		**LB237**			
Fungicide	**Log value**	**Transformed**	**Log value**	**Transformed**	**Log value**	**Transformed**	**Log value**	**Transformed**
Azoxystrobin	1.302	MC[2]	-2.269	mc[3]	1.010	MC	0.014	1.033 c[1]
Carbendazim	0.038	1.091	-0.163	0.687	0.346	2.218	0.073	1.183 c
Epoxiconazole	-0.573	0.267	-0.946	0.113	-0.298	0.504	-0.606	0.248 ab
Fluquinconazole	-1.077	0.084	-1.163	0.069	-0.212	0.614	-0.817	0.152 a
Iprodione	3.231	MC	0.338	2.178	0.651	MC	1.407	MC d
Flusilazole	-0.471	0.338	-0.839	0.145	-0.111	0.774	-0.474	0.336 b
Species mean effect	0.408	2.559 z	-0.841	0.144 x	0.231	1.702 y		

[1]Values within the same rows and columns followed by the same letters are not significantly different according to fisher's protected LSD at P=0.05.
[2]Maximum concentration (>3.0 mg a.i. / L)
[3]Minimum concentration (<0.003 mg a.i. / L)

4.2 Effect of fungicide on *in vitro* conidial germination of *Leptosphaeria maculans* conidia

There was a significant effect of both fungicide and isolate, and an interaction between them on the EC_{50} value for inhibition of conidial germination (P<0.001 for all; Table A.2). Across both *L. maculans* isolates, the least effective fungicide at inhibiting conidial germination was carbendazim, having a significantly higher EC_{50} value (>3.0 mg a.i. / L) compared with all of the other fungicides (Table 4.2; Figure 4.2). The most effective fungicide was azoxystrobin with an EC_{50} value of <0.003 mg a.i. / L, which was significantly lower than all other fungicides apart from epoxiconazole (0.028 mg a.i./ L). Fluquinconazole and flusilazole were not significantly different to epoxiconazole with a mean EC_{50} of 0.141 and 0.224 mg a.i. / L, respectively. There was a significant difference in the susceptibility of the two *L. maculans* isolates, with the conidia of isolate LM145 being significantly more susceptible to fungicide treatments compared with isolate LM183.

From the results in Table 4.2, the LM183 isolate does not appear to have been controlled by the fungicides but this is due to the EC_{50} of carbendazim exceeding the maximum value. Re-analysis of the data without the carbendazim data still showed a significant effect of isolate *(P*<0.001; Table A.3), although the mean effect of LM183 and Lm 145 on EC_{50} values reduced to 0.035 and 0.061 mg a.i. / L respectively (Table 4.3).

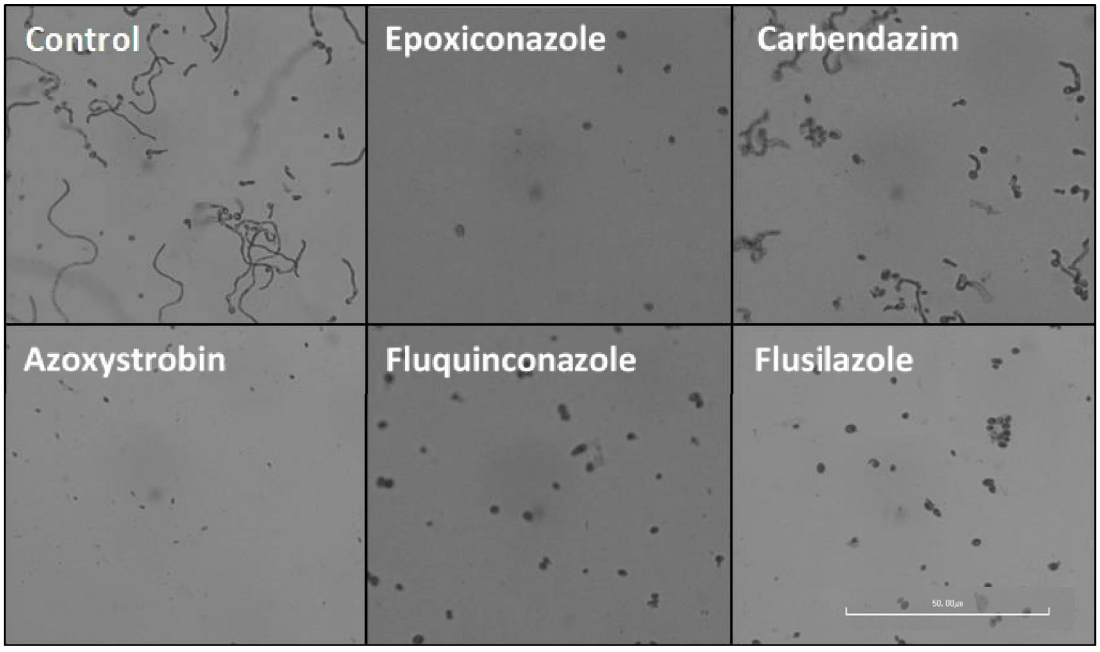

Figure 4.2 Microscope screen shots of *Leptosphaeria maculans* (LM183) conidial germination of a control treatment compared with all of the fungicide treatments at the 3.0 mg a.i / L concentration.

Table 4.2 The mean EC_{50} values (mg a.i. / L) of fungicides for their *in vitro* effect on conidial germination of two *Leptosphaeria maculans* isolates (LM183 and LM145) relative to the no fungicide control after 45 h incubation at 20°C with 12 hours light.

Fungicide	*Leptosphaeria maculans* isolate				Fungicide mean EC_{50}	
	LM183		LM145			
	Log value	Transformed	Log value	Transformed	Log value	Transformed
Azoxystrobin	-2.07	0.009	-2.52	0.003	-2.293	mc a
Carbendazim	11.64	MC[2]	1.75	MC	6.695	MC c
Epoxiconazole	-1.37	0.043	-1.73	0.019	-1.550	0.028 ab
Fluquinconazole	-0.72	0.191	-0.97	0.107	-0.846	0.141 b
Flusilazole	-0.72	0.191	-0.58	0.263	-0.651	0.224 b
Isolate mean effect	1.35	MC z	-0.81	0.155 y		

[1]Values within the same rows and columns followed by the same letters are not significantly different according to fisher's protected LSD at P=0.05.
[2]Maximum concentration (>3.0 mg a.i. / L)

Table 4.3 The mean EC_{50} values (mg a.i. / L) across all fungicides excluding carbendazim for their *in vitro* effect on conidial germination of two *Leptosphaeria maculans* isolates (LM183 and LM145) relative to the no fungicide control after 45 h incubation at 20°C with 12 hours light.

Species Mean Effect	*Leptosphaeria maculans isolate*	
	LM183	**LM145**
Log value	-1.218	-1.452
Transformed	0.061 b[1]	0.035 a

[1]Values within the same rows and columns followed by the same letters are not significantly different according to fisher's protected LSD at P=0.05.

4.3 Effect of fungicides on *in vitro* conidial germination of *Leptosphaeria biglobosa* conidia

There was a significant effect (*P*<0.001; Table A.4) of fungicide treatment on conidial germination of *L. biglobosa* isolate LB237. Azoxystrobin was significantly more effective at reducing conidial germination compared with all other fungicides, with an EC_{50} value of <0.003 mg a.i. /L (Table 4.4). The next most effective fungicide was flusilazole with a significantly lower EC_{50} than the remaining fungicides (0.938 mg a.i. /L). Carbendazim, epoxiconazole, and fluquinconazole were not effective at reducing conidial germination, all having EC_{50} values of >3.0 mg a.i. / L.

Table 4.4 The mean EC_{50} (mg a.i. / L) values of different fungicides for the *in vitro* effect on conidial germination of *Leptosphaeria biglobosa* isolate LB237 relative to the no fungicide control after 20 h incubation.

Fungicide	Azoxystrobin	Carbendazim	Epoxiconazole	Fluquin-conazole	Flusilazole	Grand mean
Log value	-3.139	1.296	2.159	0.495	-0.028	0.157
Transformed	mc[3] a	MC d	MC e	MC c	0.938 b	1.435

4.4 Fungicide application at field rates to inhibit *Leptosphaeria maculans* and *L. biglobosa* inoculation of a susceptible and resistant swede variety

All three fungicide treatments, azoxystrobin, epoxiconazole and fluquinconazole were successful in inhibiting infection from the conidial inoculation by both *L. maculans* and *L. biglobosa* with no signs of lesion development at 15 days post inoculation. In addition, no lesions developed on the cotyledons of the negative control (water inoculated) seedlings. Since no lesions developed on the fungicide or uninoculated negative control these were omitted from the statistical analysis. Lesions were observed on the cotyledons of all three positive control treatments (*L. maculans* inoculated resistant and susceptible swede cultivars and *L. biglobosa* inoculated susceptible swede cultivar) after 15 days. There was no significant effect of *Leptosphaeria* spp. (*P*= 0.71; Table A.5) or swede cultivar (P=0.85; Table A.5) on the diameter of the lesions which developed on the positive control inoculated swede seedlings (Figure 4.3). Mean diameter was 2.48 mm and 2.82 mm for the resistant and susceptible swede cultivars inoculated with *L. maculans* respectively and 3.22 mm for the susceptible swede cultivar inoculated with *L. biglobosa*. There was also no significant effect of *Leptosphaeria* spp. (*P*= 0.886; Table A.6) or swede cultivar (*P*=0.679; Table A.6) on the scores given to the lesions which developed on the positive control inoculated swede seedlings (Figure 4.4). Mean lesion score was 3 and 3.625 for the resistant and susceptible swede cultivars inoculated with *L. maculans*, respectively and 3.125 for the susceptible swede cultivar inoculated with *L. biglobosa.*

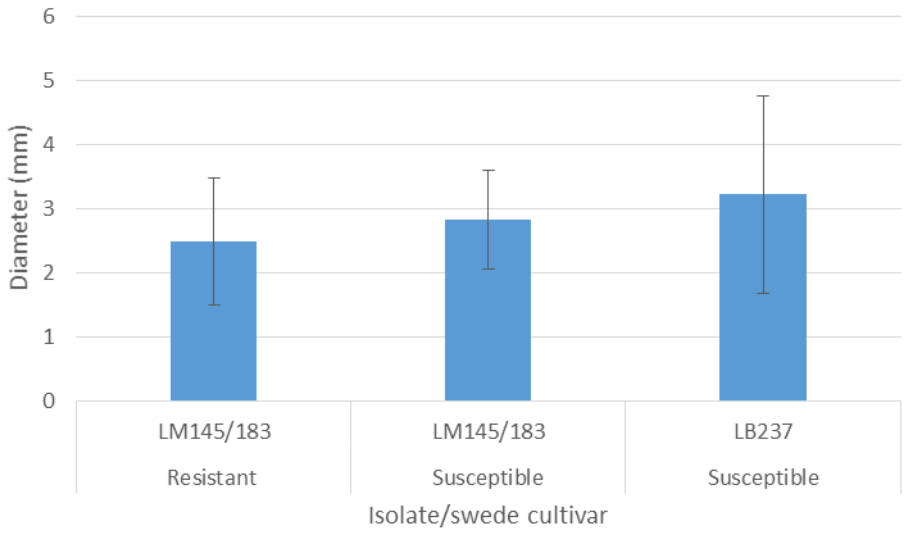

Figure 4.3 Mean diameter (mm) of the positive control treatments, resistant and susceptible swede cultivars inoculated with *Leptosphaeria maculans*. (LM145/LM183) or *L. biglobosa* (LB237) conidial suspension, with error bars representing standard error.

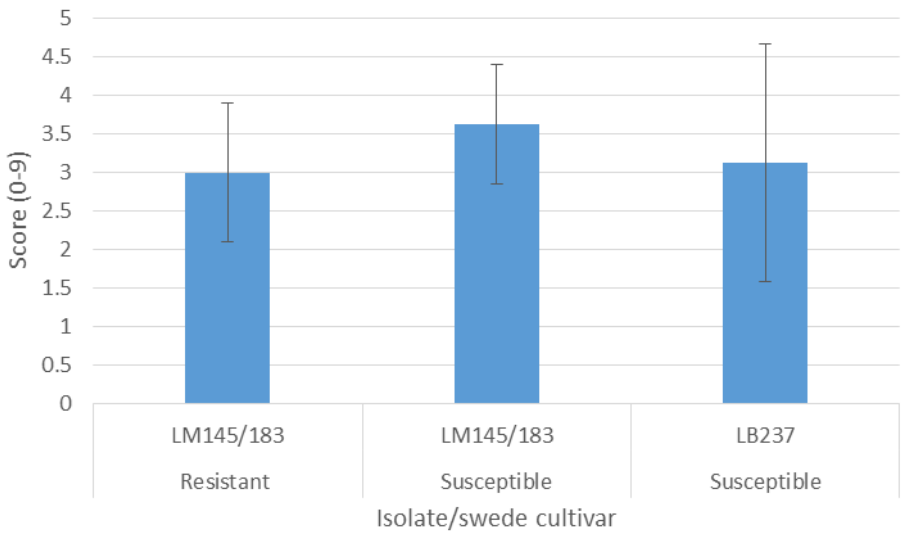

Figure 4.4 Mean lesion score of the positive control treatments, resistant and susceptible swede cultivars inoculated with *Leptosphaeria maculans* (LM145/LM183) or *L. biglobosa* (LB237) conidial suspensions. Means of 10 replicates for each treatment, with error bars representing standard error.

Colony characteristics of *L. maculans* were observed to grow on the agar from the leaf lesion tissue which developed on the positive controls inoculated with *L. maculans* (Table 4.5; Figure 4.5). Similarly, colony characteristics of *L. biglobosa* were observed to grow on the agar from the leaf lesion tissue which developed on positive controls inoculated with *L. biglobosa* (Table 4.5; Figure 4.5). No colony characteristics of *Leptosphaeria* spp. were isolated from any cotyledon tissue plated from control treatments which did not produce a lesion. Further, no colony characteristics of *L. maculans* or *L. biglobosa* were isolated from any cotyledon tissue taken from the inoculation point when plated from the fungicide or negative control treatments, confirming there was no *Leptosphaeria* spp. infection on the fungicide treated seedlings.

Table 4.5 Confirmation of Koch's postulates for seedlings inoculated with *L. maculans* (LM), *L. biglobosa* (LB) or the untreated control (water) and the different fungicide treatments, indicating the re-isolation of colony characteristics of *L. maculans* or *L. biglobosa* recovered from the cotyledon tissue plated onto potato dextrose agar.

Spray treatment	Swede cultivar	Inoculation treatment	Lesion presence/absence	Colony morphology
Water	Susceptible	LM	Presence	*L. maculans*
Water	Susceptible	LM	Presence	*L. maculans*
Water	Susceptible	LM	Presence	*L. maculans*
Water	Resistant	LM	Presence	*L. maculans*
Water	Resistant	LM	Presence	*L. maculans*
Water	Resistant	LM	Presence	*L. maculans*
Water	Susceptible	LB	Absence	Negative
Water	Susceptible	LB	Presence	*L. biglobosa*
Water	Susceptible	LB	Presence	*L. biglobosa*
Water	Resistant	Water	Absence	Negative
Water	Susceptible	Water	Absence	Negative
Epoxiconazole	Susceptible	LM	Absence	Negative
Epoxiconazole	Susceptible	LB	Absence	Negative
Epoxiconazole	Resistant	LM	Absence	Negative
Azoxystrobin	Susceptible	LM	Absence	Negative
Azoxystrobin	Susceptible	LB	Absence	Negative
Azoxystrobin	Resistant	LM	Absence	Negative
Fluquinconazole	Susceptible	LM	Absence	Negative
Fluquinconazole	Susceptible	LB	Absence	Negative
Fluquinconazole	Resistant	LM	Absence	Negative

Figure 4.5 Confirmation of Koch's postulates showing representative colonies which developed from cotyledon tissue from the different treatments plated on potato dextrose agar after 20 days incubation at 20 °C in the dark. Colonies were morphologically identified as *Leptosphaeria maculans* (1-2), *L. biglobosa* (photos 3-4) and other fungi/bacteria (photos 5-8).

After 70 days there were very little disease symtoms observed in any of the treatments with the infected cotyledons of the positive control treatments having senesced (Figure 4.6). Five plants exhibited signs of disease through death of the plant and stem lesions (Table 4.6). *Leptosphaeria* spp. was only re-isolated from one plant, a susceptible swede which had been inoculated with *L. maculans* (Table 4.6; Figure 4.7).

Table 4.6 Results for re-isolation colony characteristics of tissue samples from seedlings inoculated with *Leptosphaeria maculans* (LM) which exhibited signs of *Leptosphaeria* spp. disease development after 70 days.

Fungicide	Swede Cultivar	Inoculation treatment	Colony morphology
Epoxiconazole	Susceptible	LM	Absent
Epoxiconazole	Susceptible	LM	Absent
Water	Susceptible	LM	Absent
Water	Susceptible	LM	*L. maculans*
Water	Susceptible	LM	Absent

Figure 4.6 Appearance of the swede seedlings in the pot experiment after 70 days showing senescence of oldest leaves (Red arrows).

Figure 4.7 Confirmation of Koch's postulates showing representative colonies which developed from diseased/dead tissue on potato dextrose agar after 20 days incubation at 20 °C in the dark. Colonies were morphologically identified as *Leptosphaeria maculans* (left), and other fungi/bacteria (middle & right).

Chapter 5

Discussion

5.1 Pot experiment

The pot experiment showed that all of the fungicides tested are capable of inhibiting *L. maculans* and *L. biglobosa* conidial infection of resistant and susceptible swede varieties, proving that there is potential for the use of fungicides to control dry rot in New Zealand swedes.

This was a good indication of the potential of fungicides to control dry rot in swedes as it was in a controlled environment and allowed for uniformity of the treatments and environmental factors each unit was exposed to. It was also easy to set up and a low cost alternative to a field experiment, which were important factors for an honours project. The disadvantage of a pot experiment is that the results cannot be directly translated to how effective the fungicides would be under field conditions due to differences in rainfall, nutrient availability, pot size, temperature and fungicide application method. Watering of the plants was done by applying water to the potting mix surface under the leaves so the fungicides and fungal inoculants would not be washed off by overhead watering. This ensured the fungicides and fungal inoculants remained present on the plant but it did not simulate what happens in the field where rain may wash fungicides off the plant surface (Schilder, 2010). Variations in soil depth, texture and nutrient and water availability across a paddock and between fields are also factors not simulated in a greenhouse pot trial. Pot size may have affected the ability of the plant to grow efficiently and defend itself from the pathogen, this was not a problem for the 15 day measurements but by day 70 the pots were heavily root bound. The growth medium used in this experiment was a calculated formulation of textures and nutrients for optimum seedling growth which did not simulate the stresses that plants would encounter in the field. The plants also did not encounter water or temperature stress with bi-daily watering and temperature regulation in the greenhouse. Moisture stress and temperature fluctuations are likely to have an effect on a plants defences and the growth of fungal pathogens (West *et al*. 2001; Huang *et al.,* 2001). Spray application effects the retention, deposition and ability of the fungicide to penetrate the plant (Ratajkiewic *et al.*, 2009). The fungicides were applied using a spray bottle until runoff, this was the most effective way to keep the treatments uniform but it did not accurately simulate the boom sprays used in the field.

The single drop of conidial spore suspension applied as inoculum did not simulate the conditions in the field where the plants would be challenged by ascospore releases with each rain event (West *et al*., 2001). Due to this constant spore pressure it would be assumed that fungicides need to be

repeatedly sprayed to keep protecting the plant at a cost which cannot be warranted in forage swedes. McGee & Petrie (1979) showed that inoculation of the leaves of oilseed rape after the sixth leaf stage was too late to develop stem cankers which impacted yield loss. Further research should study this in swedes and determine at what point swedes need to be protected until new infections will not impact on yield. Number of spores used at each inoculation site (~1000) was much higher than would be found in a natural environment where maybe one or two spores will land on a particular site (Wood & Barbetti, 1977). This may mean that under field conditions these fungicides would be more effective due to lower inoculum concentrations.

In the natural environment the primary inoculum source is most commonly by ascospores with conidia causing secondary infection between the plants within the crop (West *et al*. 2001). Conidia were used in this experiment as they are easily produced under laboratory conditions in large numbers to create spore suspensions. Ascospores are much more difficult to produce as they are the sexual reproduction spores and require special conditions to be formed. This includes the presence of two compatible mating types in the stem of the host plant which have grown systemically from leaf or cotyledon infections. It is believed this process is to retain a strong gene pool of *Leptosphaeria* spp. (Travadon *et al*., 2009). Simulation of these conditions to produce ascospores in the laboratory using a sterile toothpick embedded into agar have been successful (Lob, 2014), but the number of pseudothecia produced is minimal which makes the use of ascospores in laboratory experiments difficult. In further experiments of the successful fungicides from this study, ascospore germination inhibition should be assessed to identify differences in germination inhibition of the two spore types. A field trial would be effective as this will expose the fungicides to natural spore pressure which will include ascospores.

To confirm the efficacy of the fungicides for commercial application, field experiments will be required to ensure the factors stated above are not influencing the results achieved in this experiment. It would also be beneficial to test the differences in the timings of the fungicide spray in relation to inoculation. This would give an indication of how quickly a spray needs to be applied after a spore release event, or how long after fungicide application is the fungicide still able to protect the plant from subsequent spore challenge. Another factor not considered for this experiment was the effect of the fungicides on the growth of swedes. Some fungicides effect plant processes such as epoxiconazole which reduces oilseed rape growth by inhibiting gibberellin biosynthesis (Durenne *et al*., 2017). Plant growth should be measured in ongoing experiments as any negative effects from the fungicides will impact on their suitability in commercial situations.

5.2 Fungicide efficacy

Both the *in vitro* mycelial and conidial germination inhibition experiments proved that there is varying susceptibility of *Leptosphaeria maculans* and *L. biglobosa* isolates to the fungicides tested. This highlights the importance of rigorously testing any potential fungicides against several isolates of the species to ensure they provide adequate control in the field. This would also identify if there is any risk for the potential development of resistance.

Azoxystrobin was very effective at inhibiting conidial germination (EC_{50} of mc) but was not as effective for inhibiting mycelial growth (EC_{50} of 1.033 mg a.i. / L). Novachem Manual (2017) states it is best used as a protectant fungicide which is what was seen in the results as the *in vitro* conidial germination simulates azoxystrobins ability to protect plants from conidial attack. Azoxystrobin has been shown to protect oilseed rape against *Leptosphaeria* spp. in the field by Ratajkiewic *et al*. (2009). Although, that experiment was in Poland where *L. biglobosa* is dominant over L*. maculans* it still demonstrates azoxystrobins ability to control this disease in the field. This is what was observed in the pot experiment where azoxystrobin was capable of inhibiting infection of a susceptible and resistant swede variety under conidial spore pressure.

Epoxiconazole is also described as a protectant fungicide by the Novachem Manual (2017) which supports the germination inhibition result that was found (EC_{50} of 0.028 mg a.i. /). It is also stated that it shows some control against early stages of infection which explains why it also performed moderately well in mycelial growth inhibition (EC_{50} of 0.248 mg a.i. / L). There were no studies found which have assessed epoxiconazoles efficacy to inhibit *Leptosphaeria* spp. Although, Epstein, L. (2014) stated the fungicide group which epoxiconazole belongs to (triazole) are commonly used for *L. maculans* control in Europe, suggesting epoxiconazole is likely to control *L. maculans.*

Fluquinconazole is stated to have contact, residual, disinfectant and preventative actions (Novachem Manual, 2017) which explains why it was effective at inhibiting both mycelial growth and conidial germination. Fluquinconazole is marketed as a seed treatment but when applied as a foliar spray it was effective at inhibiting infection of inoculated seedlings. Munro (2007) has shown that fluquinconazole provides very little protection as a seed treatment; although repeated studies need to confirm this. If successful, a seed treatment would reduce the cost of application as it would not require machinery for spray application and only use a small amount of the product to coat the seeds. This is important for control of *Leptosphaeria* spp. in forage swedes as they are not a high value crop.

Flusilazole was moderately effective at inhibiting both mycelial growth and conidial germination which was expected as Novachem Manual (2017) stated it stops germ tube growth and inhibits

mycelial growth of fungi. Similar results were obtained by Eckert *et al*. (2010) who found the EC_{50} for *L. maculans* and *L. biglobosa* mycelial growth inhibition was 0.27 and 0.34 mg a.i / L respectively. They also found that *L. maculans* conidial germination was completely inhibited by all the concentrations they tested and that 50% of the *L. biglobosa* growth was controlled at the higher concentration (1 mg a.i. / L). Huang *et al*. (2011) found that the fungicide Punch C which contains flusilazole and carbendazim was able to reduce the size of leaf lesions on oilseed rape inoculated with *L. maculans* but not *L. biglobosa.* This supports the results found in this study where flusilazole, as well as the other fungicides, have not inhibited *L. biglobosa* to the same level as *L. maculans*.

Carbendazim was not an effective inhibitor of mycelial growth or conidial germination which may be an indication of fungicide resistance as Novachem Manual (2017) gives warnings for resistance development in the product brief. If resistance was the cause of this failing, it would have had to develop on other crops such as oilseed rape as no fungicides are registered for dry rot of swedes in New Zealand. Eckert *et al*. (2009) found that carbendazim was an ineffective fungicide for the control of *L. maculans* and *L. biglobosa* in the UK. It was able to inhibit mycelial growth at the higher concentrations which may be due to the eradicant properties described by the Novachem Manual (2017). Huang et al. (2011) showed a mixture of flusilazole and carbendazim reduced the size of leaf lesions on oilseed rape inoculated with L. maculans; although it is likely flusilazole was the greatest contributor to the inhibition they produced due to the results seen in this study.

Iprodione was highly ineffective for the inhibition of mycelial growth so its use was not continued for the conidial germination experiment. It may have been interesting to measure its capabilities for conidial germination inhibition as the Novachem Manual (2017) stated that it inhibits the germination of spores. Although, if it cannot inhibit mycelial growth, it will not be able to protect the plants from spores which have already colonised. It is also only a contact fungicide so it would not be able to kill *Leptosphaeria* spp. infection which has penetrated the leaf tissue (Schilder, 2010).

5.3 *Leptosphaeria* spp. isolate variation

The *L. maculans* LM183 isolate was more resistant to the fungicides than the *L. maculans* LM145 isolate for both mycelial growth inhibition and conidial germination inhibition. This demonstrates the variability within the population and in this experiment it was lucky to demonstrate this with only two *L. maculans* isolates. In the future a greater number of isolates will have to be used to ensure there is a representative sample of the population.

The results of this study have indicated that *L. biglobosa* is not as susceptible to the fungicides tested compared with *L. maculans* which has been reported in several studies (Eckert *et al.,* 2010; Ratajkiewicz *et al*., 2009; Huang *et al*., 2011). Azoxystrobin was the only fungicide to exhibit a high

level of control in the *in vitro* inhibition experiments, although it only provided effective inhibition of conidial germination and not mycelial growth. Despite this, azoxystrobin, epoxiconazole and fluquinconazole were capable of inhibiting *L. biglobosa* infection of the susceptible swede variety. With only one isolate tested, no conclusions about the overall *L. biglobosa* population in New Zealand can be made but even if only some isolates are resistant they will quickly be selected for with application of the fungicide, resulting in the development of a fungicide resistant population. Further experiments should test more isolates to confirm the reduced sensitivity of *L. biglobosa* to these fungicides across the wider population of *L. biglobosa* and study its potential to become a problem. Only a very small percentage of the *Leptosphaeria* spp. isolates recovered from symptomatic brassica crops in the survey by Lob (2014) were identified as *L. biglobosa* (2.4%) but it still needs to be studied as it may become a problem in the future. If farmers begin to use fungicides to control dry rot in swedes in New Zealand there is a possibility that when the fungicides inhibit *L. maculans* growth, *L. biglobosa* will have the chance to thrive. Travadon *et al*. (2009) showed that different isolates of *L. maculans* growing on the same lesion will supress each other's systemic growth in oilseed rape, providing evidence for the theory that *L. maculans* is suppressing *L. biglobosa* growth on swedes in New Zealand. Climate change could also impact the spread of *L. biglobosa* as in some areas of the world *L. biglobosa* is the most prevalent of the two *Leptosphaeria* spp. (Karolewski *et al*., 2002). If New Zealand's climate is to significantly change it could allow *L. biglobosa* to thrive which will impact the efficacy of some fungicides for the control of dry rot.

5.4 Fungicide concentration range

A wider range of fungicide concentrations could have been used to allow for accurate EC_{50} calculations of the fungicides which gave a maximum or minimum value. This would have been useful to know what range these fungicides operate at but for the purpose of this study they were not needed. Any fungicides not performing below the concentration range tested (3.0 mg a.i. / L) should not be used in the field as the use of fungicides which require high concentrations to control the disease encourage the development of fungicide resistance, as well as increasing the application costs to the grower. Testing lower concentrations would allow us to predict how low the concentrations used in the field could be but testing this would be more applicable in the field.

5.5 Resistant and susceptible swede varieties

The two swede cultivars supplied by Agriseeds were reported to be resistant and susceptible varieties. The pot experiment showed no difference in the susceptibility of the two swede varieties to *L. maculans* with both varieties developing lesions which did not differ in size or severity. This may be due to the difference between seedling resistance and mature plant resistance. Seedling resistance is known as qualitative resistance which occurs from the seedling to mature plant stages and can be

effective as early as the cotyledon stage. It is due to major genes which interact with corresponding genes in the fungus. A plant with an R gene which corresponds to the AVR gene of the pathogen will block the virulence of that pathogen. Mature plant resistance is known as quantitative resistance which is formed once the plant is well developed and is controlled by many genes (Fernando *et al.,* 2007). This form of resistance can cause varieties which are identified as resistant in their mature stages to be susceptible at the seedling stage (Smit & Parlevliet, 1990). The seeds of the resistant cultivar which were supplied by Agriseeds are likely to have had mature resistance (Quantitative) which was not effective when the plants were inoculated at the seedling stage in this experiment. This is expected as plant breeders select for mature plant resistance which is much more difficult to manipulate but is more durable to selection pressure than seedling resistance which often fails after three to four years (Hayward *et al*., 2012). It is also possible that intraspecific competition between the *L. maculans* isolates LM183 and LM145 may have affected these results as Travadon *et al*. (2009) has shown that if different *Leptosphaeria* spp. isolates are competing for the same resources it will have a negative effect on their growth. At the 70 day assessments, the treatments which had shown lesions at day 15 no longer had any signs of infection due to the symptomatic cotyledons falling off. If the experiment was run for an extended period of time without a restriction of soil space it would have allowed for any systemic mycelium present within the plant to develop lesions in the bulb.

5.6 Conclusion

The results of this study have identified fungicides with potential to control both *Leptosphaeria maculans* and *L. biglobosa* infection of swede in New Zealand which warrant further testing under field conditions. Although the fungicides were shown to vary in their efficacy in the *in vitro* experiments they showed promising results in the pot experiment. This has proven there is potential for the use of fungicides in the control of dry rot (*Leptosphaeria* spp.) on forage swedes in New Zealand. However, due to the cost further studies are required to provide an economically effective solution which reduces yield loss. An important factor will be the number of sprays that would be required for effective control under field conditions during seedling growth and whether seed application could provide adequate control.

Appendix A

ANOVA results

Table A. 1 ANOVA results of mean EC_{50} (mg a.i. / L) values of different fungicides for the *in vitro* effect on mycelial growth of two *Leptosphaeria maculans* isolates (LM145 and LM183) and one *L. biglobosa* isolate (LB237) relative to the no fungicide control after 12 days growth on agar.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	2	27.3932	13.6966	130.03	<.001
Fungicide	5	48.2538	9.6508	91.62	<.001
Isolate.Fungicide	10	42.9604	4.2960	40.78	<.001
Residual	72	7.5841	0.1053		
Total	89	126.1916			

Table A. 2 ANOVA results of species mean effect EC_{50} (mg a.i. / L) of fungicides for their *in vitro* effect on conidial germination of two *Leptosphaeria maculans* isolates (LM183 and LM145) relative to the no fungicide control after 45 h incubation.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Fungicide	4	426.091	106.523	90.89	<.001
Isolate	1	46.954	46.954	40.07	<.001
Fungicide.Isolate	4	149.880	37.470	31.97	<.001
Residual	30	35.158	1.172		
Total	39	658.084			

Table A. 3 ANOVA results of species mean effect EC_{50} (mg a.i. / L) of fungicides for their *in vitro* effect on conidial germination of two *Leptosphaeria maculans* isolates (LM183 and LM145) relative to the no fungicide control after 45 h incubation, excluding carbendazim.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Fungicide	3	13.37246	4.45749	137.41	<0.001
Isolate	1	0.43730	0.43730	13.48	0.001
Fungicide.Isolate	3	0.40640	0.13547	4.18	0.016
Residual	24	0.77854	0.03244		
Total	31	14.9940			

Table A. 4 ANOVA results of species mean effect EC_{50} (mg a.i. / L) of fungicides for their *in vitro* effect on conidial germination of *Leptosphaeria biglobosa* isolate LB237, relative to the no fungicide control after 45 h incubation.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Fungicide	4	65.25433	16.31358	173.14	<.001
Residual	15	1.41333	0.09422		
Total	19	66.66766			

Table A. 5 ANOVA results of mean diameter (mm) of the positive control treatments, resistant and susceptible swede cultivars inoculated with *Leptosphaeria maculans* (LM145/LM183) or *L. biglobosa* (LB237) conidial suspensions

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Inoculum	1	1.72	1.72	0.14	0.710
Swede	1	0.45	0.45	0.04	0.85
Residual	21	254.92	12.14		
Total	23				

Table A. 6 ANOVA results of lesion score (0-9) of the positive control treatments, resistant and susceptible swede cultivars inoculated with *Leptosphaeria maculans* (LM145/LM183) or *L. biglobosa* (LB237) conidial suspensions

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Inoculum	1	0.188	0.188	0.02	0.886
Swede	1	1.563	1.536	0.18	0.679
Residual	21	186.750	8.893		
Total	23	188.500			

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